Surface Functionalization with Carboxylic Acids by Photochemical Microcontact Printing and Tetrazole Chemistry

Christoph Buten,[†] Sebastian Lamping,[†] Martin Körsgen,[‡] Heinrich F. Arlinghaus,[‡] Craig Jamieson,[§] and Bart Jan Ravoo[,†]*

[†]Organic-Chemistry Institute and Center for Soft Nanoscience, Westfälische Wilhelms-Universität Münster, Corrensstraße 40, 48149 Münster, Germany

[‡]Physics Institute, Westfälische Wilhelms-Universität Münster, Wilhelm-Klemm-Straße 10, 48149 Münster, Germany

[§]Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, United Kingdom

Abstract

In this paper we show that carboxylic acid functionalized molecules can be patterned by photochemical microcontact printing on tetrazole-terminated self-assembled monolayers. Upon irradiation, tetrazoles eliminate nitrogen to form highly reactive nitrile imines, which can be ligated with several different nucleophiles, carboxylic acids being most reactive. As a proof of concept, we immobilized trifluoroacetic acid to monitor the reaction with X-ray photoelectron spectroscopy (XPS). Moreover, we also immobilized peptides and fabricated carbohydrate-lectin as well as biotin-streptavidin microarrays using this method. Surface patterning was demonstrated by fluorescence microscopy and time-of-flight secondary ion mass spectrometry (ToF-SIMS).

Introduction

Functional surfaces are highly relevant in materials science, because they can be used for the fabrication of biological active surfaces as microarrays and sensors by spatial deposition of e.g. oligonucleotides, peptides, proteins and carbohydates.[1] To obtain such surfaces, they have to be chemically modified. In the field of surface chemistry, many different ways are reported to induce chemical reactions. Among these, thermally induced reactions, catalyzed reactions and photochemical reactions are versatile methods to achieve surface functionalization.[2–5] Especially the use of light is very advantageous in this field, since light can be used as an external stimulus with high resolution in space and time. Light can induce surface functionalization by various reactions, including cycloadditions with thioaldehydes generated from phenacylsulfides, Diels-Alder cycloadditions and azide-alkyne cycloadditions.[6]

A classic example for the use of light in materials science is photolithography, in which photoresists are used in the preparation of circuit boards, where a light-sensitive polymer changes its solubility upon irradiation with light. Parts of the resist can be removed, leaving the underlying material blank for a subsequent etching process. Another way to pattern substrates with light is to use photochemical reactions to covalently immobilize molecules on a surface. One powerful method is the thiol-ene reaction, where a surface-bound alkene or thiol reacts with its counterpart using a (photo-) initiator to induce the radical reaction.[7] By this method, biotin and galactoside microarrays[4,8] could be fabricated as well as multifunctional surfaces[9] and enzyme microarrays.[10]

Along with the use of photomasks, microcontact printing can be a method of choice to prepare patterned surfaces in (sub-) micrometer-sized resolutions. In microcontact printing, a patterned elastomer stamp is used to create surface functionalization. The stamp is incubated with a solution of the molecule to be immobilized, named ink, and placed on the substrate. In conventional microcontact printing, the ink adheres to the substrate due to a combination of chemisorption and/or physisorption.[11] In microcontact chemistry, often also referred to as reactive microcontact printing, a chemical reaction between the ink and the substrate is induced by an added reagent, a catalyst or an external stimulus, and the ink is covalently bound to the surface exclusively in the areas of contact between stamp and surface.[12] The main advantages of this approach are its low-cost and fast access towards large functionalized areas.[13] Because of its chemical inertness and transparency, PDMS as the material for elastomeric stamps gained a lot of interest for photochemical reactions in the field of surface chemistry.[14,15] The main disadvantage of the thiol-ene reaction in microcontact chemistry is the need of relatively high photoinitiator concentrations in order to achieve sufficiently fast reaction rates.

An example for initiator-free photochemical reactions are the additions of nucleophiles to nitrile imines photolytically generated from tetrazoles. Nitrile imines are highly reactive 1,3-dipoles that can be trapped by various different dipolarophiles in (cyclo-) addition reactions, such as alkenes, alkynes and thiols (Figure 1), but also additions with amines and carbonyls are reported.[16,17] In 1967 it was found by HUISGEN, that nitrile imines can be generated from tetrazoles upon irradiation with ultraviolet light.[18] Tetrazoles are five-membered heterocycles consisting of four nitrogen atoms and one carbon atom. When irradiated, tetrazoles eliminate nitrogen and form the corresponding nitrile imine. LIN et al. showed that the rate constant of reactions of 2,5-diaryl tetrazoles with alkenes depends on the substituents on the aryl rings. Electron donating substituents were found to accelerate the addition, especially when the substituent is bound to the *N*-aryl ring.[19] The nucleophilic addition of thiols to nitrile imines has been used in polymer-polymer conjugation and site-selective surface functionalization.[20] BARNER-KOWOLLIK and coworkers used the cycloaddition of maleimides to nitrile imines to ligate macromolecules onto biosurfaces[21] and to create photoswitchable surfaces by using azobenzene bearing dipolarophiles.[22] Although tetrazole photoclick chemistry was described as bioorthogonal multiple times[23–25] it was found by YAO that nitrile imines readily react with various biological nucleophiles and therefore disproved this statement.[26] We recently published a straightforward method to pattern tetrazole-terminated SAMs with various different alkenes, alkynes and thiols by photochemical microcontact printing. Using this strategy it was possible to grow polymers by immobilization of a maleimide-functionalized ATRP-initiator and to prepare a biotin-streptavidin microarray by printing a biotin-tagged thiol.[27]

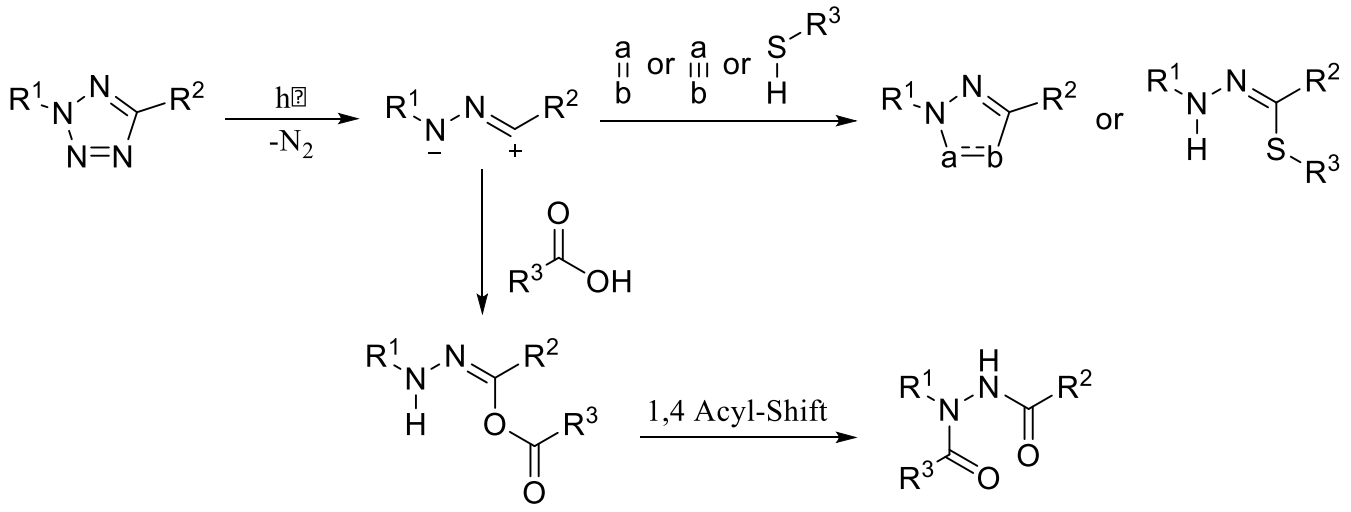

Figure 1. Reaction pathway of tetrazoles upon irradiation to nitrile imines and subsequent (cyclo)-addition of alkenes, alkynes and thiols, as well as addition of carboxylic acids and subsequent 1,4 acyl-shift.

In 1985 HEIMGARTNER et al. described the nucleophilic addition of carboxylic acids to nitrile imines to yield hydrazides (Figure 1).[28] This reaction has been used to synthesize single chain polymer nanoparticles by irradiating a polyacrylic acid bearing tetrazole moieties.[29]

In the current work, we investigated the nucleophilic addition of carboxylic acids to nitrile imines generated from tetrazole-terminated SAMs by photochemical microcontact printing. The main advantage of this approach is that many biorelevant molecules can directly be immobilized, since carboxylic acid moieties are very abundant in a variety of different biomolecules. With this fast and cheap method, tetrazole-terminated SAMs can easily be patterned with many natural and synthetic carboxylic acids, whereby site selectivity can only be guaranteed when no further intramolecular nucleophile is present. The success of the immobilization was proven with fluorescence microscopy, X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary

ion mass spectrometry (ToF-SIMS). With this method, it was possible to prepare patterns of a fluorescent peptide and arrays for the recognition of streptavidin and the lectins ConA and PNA.

Experimental Section

PDMS Stamp Preparation. PDMS stamps were prepared using a commercially available kit (SYLGARD® 184 silicone elastomer kit) from DOW CORNING. The PDMS oligomer and the curing agent were mixed in a 10:1 ratio and poured on a silicon master. The mixture was degassed *in vacuo* and cured at 80 °C over night.

Tetrazole SAM Preparation. Glass slides or silicon wafers were cut in pieces of approximately 1.4 cm x 2.6 cm and cleaned by successive sonication in *n*-pentane, acetone, ethanol and MilliQ water. These substrates were immersed in a freshly prepared piranha solution (conc. H_2SO_4:H_2O_2 (30%) = 3:1, v:v). After 30 min, the activated substrates were extensively washed with MilliQ water and dried in a stream of argon, before they were put in a freshly prepared solution of 11-bromoundecyltrichlorosilane in toluene (0.1 Vol%) for 40 min. The surfaces were sonicated in toluene, washed with ethanol and dried in a stream of argon. The 11-bromoundecyl terminated substrates were immersed in 20 mL of a 10 mM solution of tetrazole **1** in DMF saturated with K_2CO_3. The substitution was performed at 80 °C for 16 h. The surfaces were cleaned by sonication in water (2x), dichloromethane (DCM) and ethanol and dried in a stream of argon. These surfaces were used for photochemical microcontact printing.

Functionalization of Surfaces by Photochemical Microcontact Printing. Before use, PDMS stamps were oxidized by a treatment with ozone in an UV ozonizer for 55 min. The hydrophilic stamps were incubated with 40 µL of the required ink solution (25 mM) for 1 min. Subsequently, the stamps were dried in a stream of argon and placed on the tetrazole surface. The samples were placed on a mirror plate in order to reflect the light and irradiated with an UV lamp ($\lambda_{em.max}$ = 320 nm,

Compact UV Sun 20 W, Lucky Reptile) for 15 min in order to induce the reaction. The stamp was lifted off and the surfaces were cleaned by sonication in water and ethanol.

Protein Binding Studies with Streptavidin. After printing of biotin on tetrazole-SAMs, a few drops of a 100 nM solution of rhodamine labeled streptavidin in HEPES buffered saline (HBS, pH = 7.4) containing 0.02% v/v of Polyoxyethylen(20)-sorbitan-monolaurat (Tween 20; to avoid unspecific binding) were added onto the patterned substrates. After 30 min, the surfaces were thoroughly rinsed with HBS (pH = 7.4; containing 0.02% v/v Tween 20) and water and were carefully dried.

Protein Binding Studies with ConA and PNA. After printing of carbohydrates on tetrazole-SAMs, a few drops of a solution of BSA (3 wt%) in phosphate buffered saline (1xPBS, pH = 7.5) were added onto the patterned substrates to avoid unspecific binding. After 30 min, the surfaces were washed with PBS without BSA. Subsequently the substrates were incubated with a solution of the fluorescently labeled lectin (TRITC-ConA or FITC-PNA, c = 20 μg/mL) in HBS (20 mM HEPES, pH = 7.4, 150 mM NaCl, 1 mM $CaCl_2$). For ConA the buffer also contained 1 mM $MnCl_2$. After 30 min, the surfaces were rinsed with the same buffer (without lectin) and water and were carefully dried.

Results and discussion

The tetrazole-modified substrates used for the experiments were prepared by first immersing freshly activated glass- or silicon surfaces in a solution of 11-bromoundecyltrichlorosilane in toluene (1 vol%) for 30 min at room temperature. So prepared bromine-terminated SAMs were immersed in a 10 mM solution of tetrazole **1** in *N*,*N*-dimethylformamide (DMF) saturated with K_2CO_3 (for synthesis see Supporting Information). Tetrazole **1** was chosen because of its electron donating group on the *N*-aryl ring and the resulting high reactivity of the generated nitrile

imine.[19] Since PDMS is sufficiently transparent at the tetrazole absorption maximum at 290 nm (Figure 1 A), it is suitable for photochemical microcontact printing. The nucleophilic substitution of bromide with tetrazole **1** was complete after 16 h at 80 °C as shown by XPS analyses of the substrates before and after the reaction (Figure SI 2 of the Supporting Information). Vanishing of the Br3d signal at 70.6 eV and the simultaneous rise of two N1s signals at 400.4 and 402.9 eV indicates the successful formation of a tetrazole-terminated SAM. The stronger signal at 400.4 eV is assigned to the tetrazole core (-N=N-), while the weaker signal at 402.9 eV can be assigned to positively charged nitrogens.[21] Also the decrease of the surface contact angle from 112° to 80° suggests the formation of a tetrazole-terminated SAM (Figure SI 1 of the Supporting Information). To test whether the surfaces react to light, they were irradiated at 320 nm for 60 min and the N1s signal was compared with the non-irradiated sample. The decrease of the signal by a factor of two is in agreement with the assumption that two of the four nitrogen atoms in the heterocyclic scaffold are eliminated during the formation of nitrile imines (Figure SI 2 of the Supporting Information).

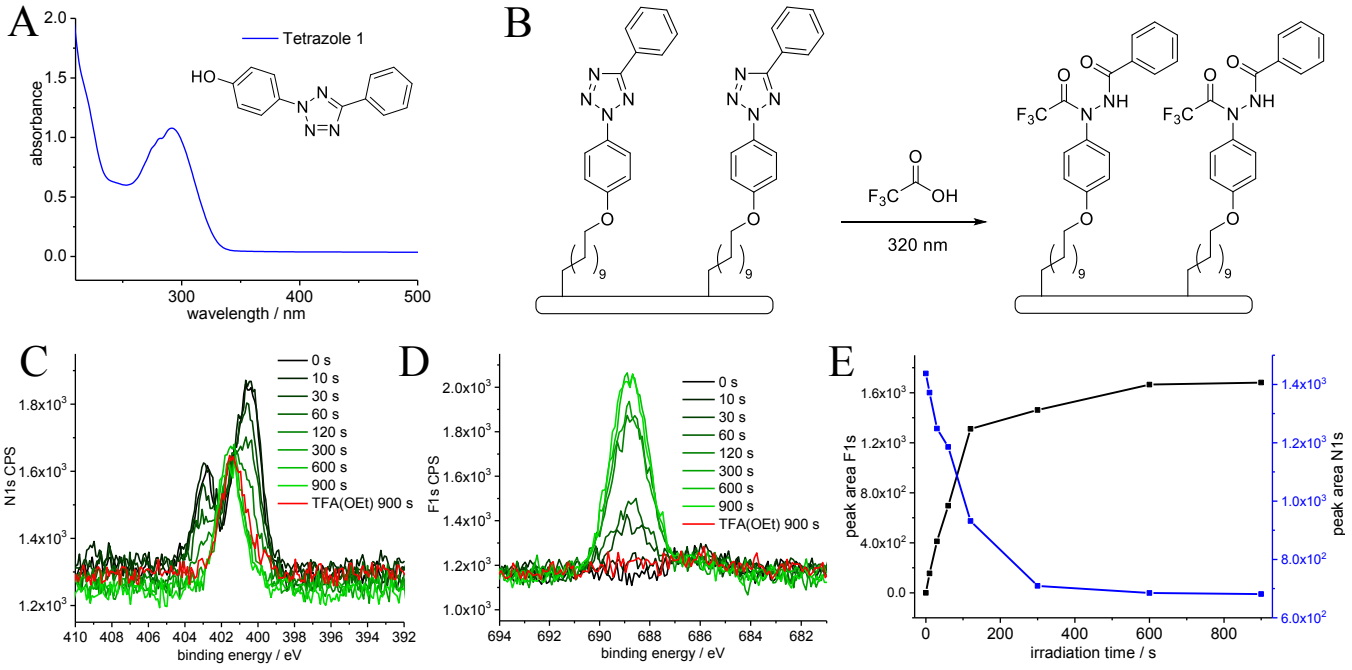

Figure 2. (A) Absorbance spectrum of tetrazole **1**, (B) on-surface reaction of TFA with surface-bound tetrazole **1**, (C) N1s signal in XPS spectra of surfaces after μCP with TFA with irradiation times between 0 s and 900 s (black to green) and with TFA(OEt) after 900 s (red), (D) F1s signal in XPS spectra of surfaces after μCP with TFA with irradiation times between 0 s and 900 s (black to green) and with TFA(OEt) after 900 s (red), and (E) peak areas of the N1s signals and F1s signals from (C) and (D) after irradiation times between 0 s and 900 s.

As a first test, we used trifluoroacetic acid (TFA) for microcontact printing on tetrazole-terminated SAMs (Figure 2 B). Because of its high fluorine content, TFA could easily be detected by XPS measurements. To this end, a flat stamp was covered with a 25 mM solution of TFA in EtOH, dried and placed on the surface. Irradiation at 320 nm for 15 min induced the reaction. The XPS spectrum after the reaction shows a large F1s signal at 688.7 eV, indicating the successful deposition of TFA. This signal can be assigned to the CF_3 groups of the hydrazide formed on the surface.[30] Control experiments without irradiation show no fluorine signal, proving that the reaction is induced by the photolytic generation of nitrile imines (Figure 2 D). The reaction with TFA was also used to investigate the kinetics of the reaction. This is important to find the right printing conditions concerning a high surface functionalization with at the same time minimum stress. To this end time-dependent XPS measurements after μCP with TFA were used. The irradiation time was varied from 0 s to 900 s. After short exposure of 10 s the peak area of the N1s signal decreased slightly while the F1s signal began to rise. Longer irradiation led to a further increasing F1s signal while the N1s signal decreased and shifted to slightly higher binding energies (401.4 eV), until after approximately 300 s significant changes could not be observed anymore (Figure 2 C-E). In total the area of the N1s peak decreased by a factor of two

from 1438 to 681 and correlates with the assumption that during the irradiation process two of the four nitrogen atoms from the tetrazole scaffold are eliminated. The F1s peak area in total increased from 0 to 1682. Taking a N/F ratio of 2/3 of the formed product on the surface after reaction and the different relative sensitivity factors for XPS of N and F in account, the yield was calculated as 71%. Qualitatively the same results for the kinetics were obtained after contact angle measurements of surfaces when glucuronic acid was printed. The contact angle decreased from 79.6° to around 64° after 300 s (Figure SI 3 of the Supporting Information). To avoid undesired functionalization through unreacted tetrazoles, the irradiation time for later applications was set to 15 min. We conclude that carboxylic acid functionalized molecules can be immobilized on tetrazole SAMs in high densities within 300 s by photochemical μCP. An additional control experiment using acid-protected trifluoroacetic acid ethyl ester showed no significant F1s signal (Fig. 2 D red line) after 900 s irradiation. The N1s signal showed the same behavior as other irradiated samples (i.e. loss of nitrogen). This control experiment clearly shows that surface conjugation occurs via reaction of the nitrile imine and the carboxylic acid.

An important class of natural carboxylic acids are amino acids and peptides. For an easy detection we synthesized the rhodamine (Rh) tagged model peptide RhGGG (Fig. 3 A) by solid phase peptide synthesis and used it in microcontact printing. As expected, when the peptide was printed using a structured stamp, fluorescent patterns with the same features as the used stamps could be observed by fluorescence microscopy (Fig. 3 B). Also in this case control experiments show no strong fluorescence without UV-irradiation (Fig. 3 C), indicating that photolytically generated nitrile imines are responsible for the immobilization of the peptide. Intensity profiles

reveal that with irradiation a by a factor of 20 more intense fluorescence can be observed (Fig. 3 D).

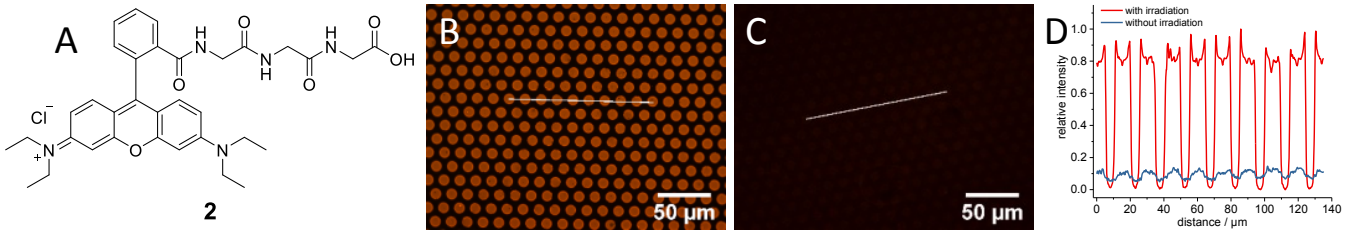

Figure 3. Analyses of peptide **2** printed on a **1**-SAM. (A) Chemical structure of the rhodamine-labeled peptide **2**, (B) Fluorescence microscopy image after μCP with 15 min UV irradiation (stamp structure: 10 μm dots spaced by 5 μm, 15 min irradiation), (C) fluorescence microscopy image after μCP without UV irradiation (stamp structure: 10 μm dots spaced by 5 μm, 15 min irradiation), and (D) intensity profiles of (B) and (C) (stamp structure: 10 μm dots spaced by 5 μm).

We further chose (+)-biotin as a biological relevant ink for microcontact printing, since it inherently exhibits a carboxylic acid moiety and could thus be easily immobilized by this method. Because of its well-known strong affinity to streptavidin, it would be a very easy access to biotinylated surfaces. We recently showed the possibility to immobilize biotin-thiol on tetrazole-terminated SAMs by the addition of a biotin-tagged thiol to nitrile imines. In this experiment, we demonstrate the immobilization without previous derivatization. To this end, we used a 25 mM solution of biotin in DMSO as ink. Successful immobilization can be indicated by condensation experiments. Irradiated samples show a preferential water condensation along the printed pattern, whereas the non-irradiated sample only shows unspecific droplet formations (Figure 4 D, E). We further analyzed the prepared substrates by ToF-SIMS. The irradiated sample shows characteristic secondary ions reproducing the chemical composition of biotin in

the shape of the given stamp structure. In contrast, ToF-SIMS measurements of the non-irradiated sample show no characteristic end group fragments of biotin. When analogously prepared substrates were incubated with rhodamine-labeled streptavidin, the given stamp structure could be observed as a red fluorescent pattern. These observations clearly show that biotin is indeed bound to the surface and is moreover present in its biologically active conformation with the acid functionality reacting with the generated nitrile imine.

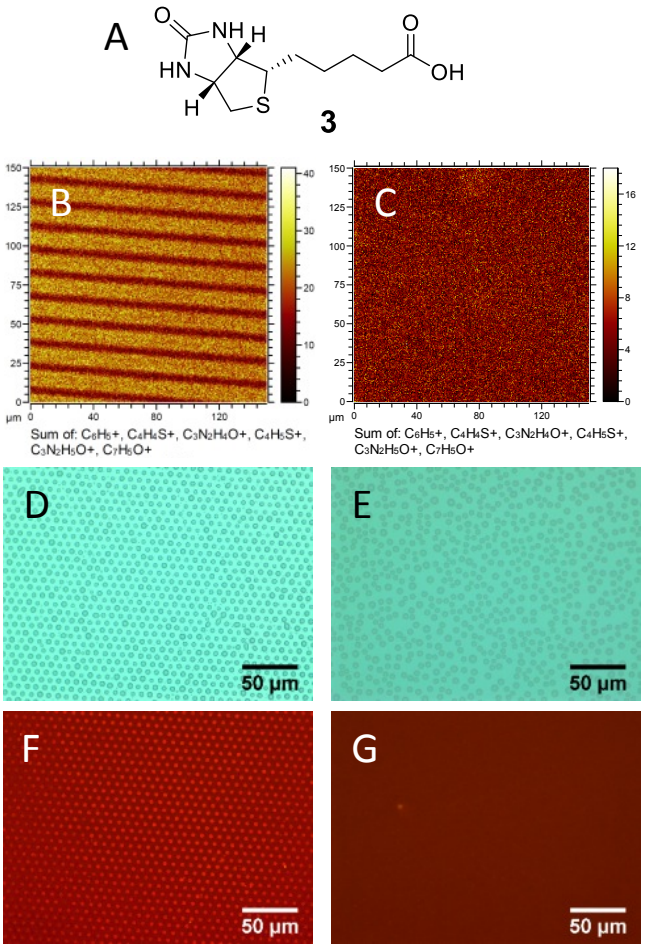

Figure 4. Analyses of biotin **3** printed on a **1**-SAM. (A) Chemical structure of (+)-biotin **3**. (B) ToF-SIMS measurement after μCP with and (C) without UV-irradiation (stamp structure: 10 μm stripes spaced by 5 μm, 15 min irradiation). (D) Light microscopy image after μCP with and (E)

without UV-irradiation. (F) Fluorescence microscopy image after μCP with and (G) without UV-irradiation and subsequent incubation with rhodamine-labeled streptavidin (stamp structure: 5 μm dots spaced by 3 μm).

In a similar approach, we synthesized two carbohydrate-acid derivatives, namely mannose-acid **4** and galactose-acid **5** for the immobilization on tetrazole-terminated SAMs. Successful deposition by photochemical microcontact printing was again analyzed by ToF-SIMS. In the areas of contact between stamp and surface, characteristic end group fragments of carbohydrates could be detected. To investigate, whether the carbohydrates still possess their lectin-binding properties, the substrates were incubated with buffered solutions of rhodamine-labeled ConA (Rh-ConA, for mannose) and fluorescein-labeled PNA (FITC-PNA, for galactose). Indeed, fluorescence microscopic analyses showed fluorescent patterns in the shape of the used stamp structure. Also in this case the non-irradiated samples showed no affinity towards the corresponding lectin. Additionally, negative control experiments where the wrong lectin was employed to either carbohydrate ligand showed no specific binding (Figure SI 4 of the Supporting Information). These observations indicate that the carbohydrates are bound to the surface with their carboxylic acid functionality and the carbohydrate scaffold is accessible for the lectin to be recognized.

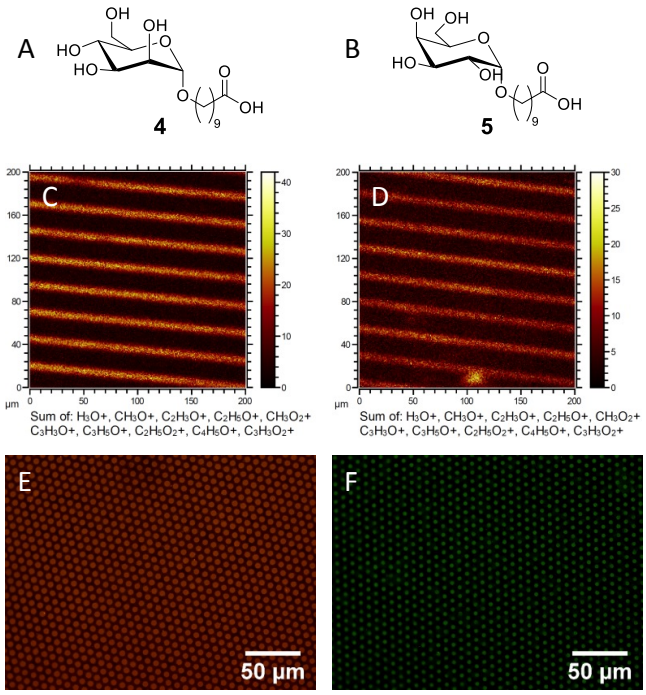

Figure 5. Analyses of carbohydrates **4** and **5** printed on a **1**-SAM. (A) Chemical structure of mannose acid **4**. (B) Chemical structure of galactose acid **4**. (C) and (D) ToF-SIMS measurements after μCP of **4** and **5** (stamp structure: 5 μm stripes spaced by 15 μm, 15 min irradiation). (E) Fluorescence microscopy image after printing of **4** and subsequent incubation with Rh-ConA, and (F) fluorescence microscopy image after printing of **5** and subsequent incubation with FITC-PNA (stamp structure: 5 μm dots spaced by 3 μm, 15 min irradiation).

Conclusion

In summary, we report a straightforward method to prepare tetrazole-functionalized surfaces that could be printed with a variety of different carboxylic acid derivatives by photochemical generation of nitrile imines. Employing this cheap and fast method, we demonstrated a versatile and easy way to immobilize biomolecules bearing this common functional group. We unambiguously proved the formation of a covalent bound by XPS, Tof-SIMS and fluorescence microscopy. Using this approach, we fabricated biotin-streptavidin and carbohydrate arrays that

show the merit of this reaction for the preparation of microarrays. Because of the abundance of carboxylic acid functionalities in many different biological molecules, this procedure may be a powerful strategy compared to other patterning and deposition techniques.

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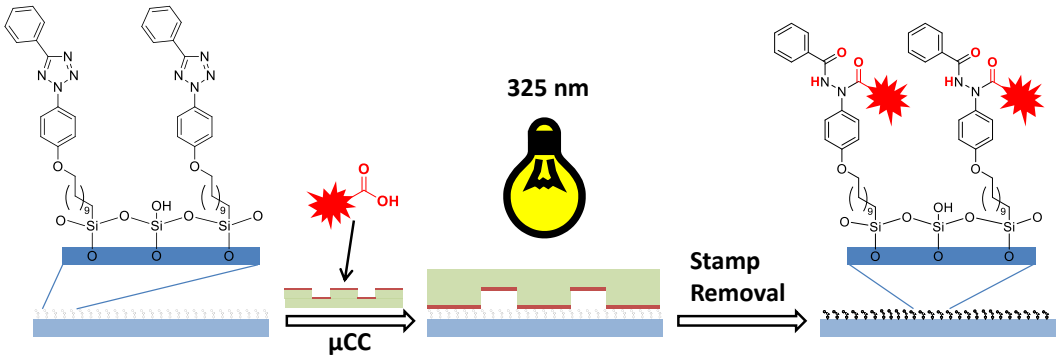
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